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Biochimica et Biophysica Acta 1590 (2002) 27–40

BIOCHIMICA ET BIOPHYSICA ACTA

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Characterisation of two *14-3-3* genes from *Trichoderma reesei*: interactions with yeast secretory pathway components

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Received 30 July 2001; received in revised form 29 January 2002; accepted 21 March 2002

Abstract

The 14-3-3 proteins are highly conserved, ubiquitously expressed proteins taking part in numerous cellular processes. Two genes encoding 14-3-3 proteins, *ftt1* and *ftt2*, were isolated and characterised from the filamentous fungus *Trichoderma reesei*. FTTI showed the highest sequence identity (98% at the amino acid level) to the *Trichoderma harzianum* protein Th1433. FTTII is relatively distinct from FTTI, showing approximately 75% identity to other fungal 14-3-3 proteins. Despite their sequence divergence, both of the *T. reesei ftt* genes were equally able to complement the yeast *bmh1 bmh2* double disruption. The *T. reesei ftt* genes were also found to be quite closely linked in the genomic DNA. A C-terminally truncated version of *ftt1* (*ftt1*Δ*C*) was first isolated as a multicopy suppressor of the growth defect of the temperature-sensitive yeast secretory mutant *sec15-1*. Overexpression of *ftt1*Δ*C* also suppressed the growth defect of *sec2-41*, *sec3-101*, and *sec7-1* strains. Overexpression of *ftt1*Δ*C* in *sec2-41* and *sec15-1* strains could also rescue the secretion of invertase at the restrictive temperatures, and overexpression of full-length *ftt1* enhanced invertase secretion by wild-type yeast cells. These findings strongly suggest that the *T. reesei ftt1* has a role in protein secretion.

Keywords: 14-3-3; *Trichoderma reesei*/*Hypocrea jecorina*; Exocytosis

1. Introduction

The 14-3-3 proteins are highly conserved and ubiquitously expressed small acidic proteins. They exist as multiple isoforms in mammals and plants while two isoforms appear to be present in fungal organisms [1–3]. The 14-3-3 proteins are involved in numerous cellular processes, for example cell cycle and survival regulation [4,5], transcriptional control [6,7], mediation of interactions between components of signal transduction pathways [8], and modulation of enzyme activities [9] and nutrient-regulated pathways [10,11]. Their exact mode of action is not known, but it has been suggested that 14-3-3 proteins control the function of their target proteins by adjusting their stability [1], activity, or intracellular localisation [12,13].

In addition to the above controlling functions, the 14-3-3 proteins have also been reported to be involved in vesicle trafficking in both lower and higher eukaryotes. In mammals, a mixture of 14-3-3 isoforms from brain cytosol reactivated the Ca^{2+}-dependent exocytosis in permeabilised adrenal chromaffin cells [14]. This stimulation was due to reorganisation of the cortical actin barrier [15] and could be related to increasing secretory vesicle availability during the ATP-dependent priming stage of exocytosis [16]. Similarly, the *Drosophila* 14-3-3 protein, Leonardo, has been suggested to regulate the releasable pool of synaptic vesicles [17]. In another experimental system, a kinesin-like motor protein involved in the retrograde trafficking between the Golgi complex and endoplasmic reticulum (ER) was found to associate with 14-3-3 proteins [18]. Furthermore, the exit of invariant chain p35 of major histocompatibility complex class II (MHCII) from ER is regulated by its phosphorylation and subsequent binding with 14-3-3 proteins [19]. In *Saccharomyces cerevisiae*, overexpression of the *14-3-3* homologue, *BMH2*, is able to rescue the viability of the clathrin heavy chain gene deletion mutant [20], and dominant-negative alleles of Bmh2p have been shown to disturb polarised exocytosis [21]. The 14-3-3 proteins thus appear to be involved in multiple steps of vesicular trafficking: in anterograde and retrograde transportation as well as in protein exit from the ER.

We report here the isolation of a C-terminally truncated *14-3-3* cDNA (*ftt1*Δ*C*) of the filamentous fungus *Trichoderma reesei* as a multicopy suppressor of yeast temper-

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0167-4889/02/$ - see front matter
PII: S0167-4889(02)00197-0

ature-sensitive secretion mutation, *sec15-1*. A second *14-3-3* gene, *ftt2*, was cloned from this fungus by hybridisation with *ftt1ΔC*. Results are presented on the complementation of a yeast *bmh1 bmh2* disruptant strain and on genetic interactions of the truncated *ftt1* cDNA with multiple components of the yeast secretory pathway.

2. Materials and methods

2.1. Strains and culture conditions

The *Escherichia coli* strains used as plasmid hosts were DH5α (F$^-$ *end*A1 *hsd*R17(r_κ^-, m_κ^+) *sup*E44 *thi*-1 λ$^-$ *rec*A1 *gyr*A96 *rel*A1 Δ(*arg*-F-*lac*ZYA)U169 ϕ80*lac*ZΔM15), and XL1-Blue MRF′ (Δ(*mcr*A) 183 Δ(*mcr*CB-*hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F′ *pro*AB *lac*I^{q}ZΔM15 Tn10 (*Tet*r)]). The yeast strains used are listed in Table 1. *T. reesei* strain Rut C30 [22] was used in Southern and Northern analyses.

The yeast strains were grown in YPD (2% bactopeptone, 1% yeast extract, and 2% glucose), in synthetic complete media lacking uracil (SCD-Ura), leucine (SCD-Leu), or tryptophan (SCD-Trp) with 2% glucose, or in yeast minimal medium [23]. *T. reesei* was grown in shake flasks in *Trichoderma* minimal medium [24] with 2% glucose for Southern analysis. For Northern analysis, it was grown in carbon-limited chemostat cultures with lactose feed and dilution rates from 0.02 to 0.07 [25].

2.2. Plasmids and nucleic acid methods

The full-length *T. reesei* cDNAs of *ftt1* and *ftt2* were isolated from λZAP-cDNA library [26]; the corresponding yeast expression clones and genomic clones were picked up from a cDNA library [27] constructed in a multicopy plasmid containing *URA3* as the selection marker [28] and from a *T. reesei* cosmid library [29], respectively, as described [30]. The plasmids were denoted as follows: pTV*ftt1* and pTV*ftt2*, the λZAP-cDNA library-derived plasmids, pTV*PGK-ftt1* and pTV*PGK-ftt2*, the yeast expression plasmids, and pMS49, the yeast expression plasmid containing truncated *ftt1* (see below). For yeast complementation experiments, the *T. reesei* cDNAs of *ftt1* and *ftt2* were released from their yeast expression plasmids by digestion with restriction enzymes *Asp*718 and *Sma*I, blunt ended and cloned between the *ADH1* promoter and *CYC1* terminator in pYcDE-2 vector (Benjamin Hall, Department of Genetics, University of Washington, Seattle, USA), which contains *TRP1* as the selection marker. The resulting plasmids were designated as pTV*ADH-ftt1* and pTV*ADH-ftt2*, respectively. The cloning of truncated *ftt1* into this vector did not succeed and therefore a major part of the *URA3* gene was deleted from the pMS49 plasmid by digestion with restriction enzymes *Acc*I and *Eco*RV and replaced by the *TRP1* gene. This plasmid is denoted pTV*PGK-ftt1ΔC(TRP1)*.

Table 1
Genotypes and sources of *S. cerevisiae* strains used in this study

Strain	Genotype	Source
NY13	*MAT**a** ura 3-52*	P. Novick, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510, USA
NY3	*MAT**a** sec1-1 ura3-52*	P. Novick
NY24	*MAT**a** sec1-11 ura3-52*	P. Novick
NY770	*MATα sec2-41 ura3-52 leu2-3,112*	P. Novick
NY772	*MAT**a** sec3-2 ura3-52 leu2-3,112*	P. Novick
BHY44	*MATα sec3-101 (psl1-1) ura3 leu2 his3*	B.K. Haarer, Department of Anatomy and Cell Biology, University of Michigan, Ann Arbor, MI 48109, USA
NY774	*MATα sec4-8 ura3-52 leu2-3,112*	P. Novick
NY776	*MATα sec5-24 ura3-52 leu2-3,112*	P. Novick
NY778	*MATα sec6-4 ura3-52 leu2-3,112*	P. Novick
Sf821-8A	*MAT**a** sec7-1 ura3-52 leu2-3,112 his4-580 trp1-289*	R. Schekman, Department of Biochemistry, University of California, Berkeley, CA 94720, USA
NY780	*MATα sec8-9 ura3-52 leu2-3,112*	P. Novick
NY782	*MAT**a** sec9-4 ura3-52 leu2-3,112*	P. Novick
NY784	*MAT**a** sec10-2 ura3-52 leu2-3,112*	P. Novick
NY786	*MAT**a** sec15-1 ura3-52 leu2-3,112*	P. Novick
HS33-1	*MATα sec15-1 ura3-52 leu2-3,112 trp1-(HindIII) his4-260 ade2-1*	S. Keränen
BY55	*MATα sec17-1 ura3-52*	P. Brennwald, Department of Cell Biology, Weill Medical College of Cornell University, New York, NY 10021, USA
mBY12-6D	*MATα sec18-1 ura3-52 leu2-3, 112 trp1-289*	R. Schekman
NY1213	*MATα sec19-1 ura3-52 leu2-3,112*	P. Novick
H1152	*MAT**a** sso2-1 ura3-1 leu2-3,112 trp1-1 sso1::HIS3*	S. Keränen
GG583	*MAT**a**/MATα leu2-3,112/leu2-3,112 ura3-52/ ura3-52 trp1-92/trp1-92 his4/+ bmh1::LEU2/+ bmh2::URA3/+*	G.P.H. van Heusden, Institute of Molecular Plant Sciences, Leiden University, NL-2333 AL Leiden, The Netherlands

Standard methods [30] were used in nucleic acid work unless otherwise stated. For Southern analysis, 5 μg of *T. reesei* DNA isolated with the Easy DNA kit (Invitrogen)

was digested with restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, or *Xho*I, and DNA fragments were separated in an 0.7% agarose gel by electrophoresis and blotted onto Hybond N membrane (Amersham). The membranes were hybridised with ^{32}P-labelled *ftt1ΔC* cDNA at 45, 50, 55, 60, and 65 °C in a mix containing 6 × SSC, 5 × Denhardt's, 0.5% SDS, 100 μg/ml herring sperm DNA. The membranes were washed in 2 × SSC for 30 min at room temperature and in 3 × SSC, 0.1% SDS for 30 min each at the hybridisation temperature. Stringent washings were performed for the filter hybridised at 65 °C.

For cosmid Southern analysis, 2 μg DNA of each cosmid (F.1 and H.2) was digested with restriction enzymes *Eco*RI, *Hin*dIII, *Pst*I, *Sph*I, or *Xho*I. The DNA fragments were separated in 0.8% agarose gel and blotted onto Hybond N membrane (Amersham). The membranes were hybridised with ^{32}P-labelled fragments from the 3′ flanking regions of *ftt1* (135 bp) and *ftt2* (294 bp) in a mix containing 6 × SSC, 5 × Denhardt's, 0.5% SDS, 50% formamide, 100μg/ml herring sperm DNA at 42 °C. The membranes were washed in 2 × SSC for 45 min at room temperature and in 0.1 × SSC, 0.1% SDS for 30 min at 68 °C.

For Northern analysis, 5 μg of *T. reesei* RNA isolated with the Trizol kit (Gibco BRL) was separated in an agarose gel and blotted onto Hybond-N membrane, which was hybridised at 42 °C in 50% formamide, 10% dextran sulfate, 1 M NaCl, 1% SDS. The membrane was washed first in 5 × SSPE for 30 min, then in 1 × SSPE, 0.1% SDS and in 0.1 × SSPE, 0.1% SDS at 42 °C. The same fragments produced by PCR as in cosmid Southern hybridisation were used as probes. The *ftt1* and *ftt2* signals were quantified with the Phosphoimager S1 (Molecular Dynamics) and normalised with respect to signals obtained with *gpd1* encoding glyceraldehyde phosphate dehydrogenase.

Sequencing was performed with dideoxy-chain termination with Sequenase (USB) and sequence-specific primers.

2.3. Yeast complementation

A diploid yeast strain, GG583, having *bmh1::LEU2 bmh2::URA3* double disruption (a gift from G.P.H. van Heusden, Leiden University, The Netherlands) was transformed with the pTV*ADH-ftt1* or the pTV*ADH-ftt2* plasmid for overexpression of *ftt1* or *ftt2*, respectively, from the *ADH1* promoter. For overexpression of *ftt1ΔC*, this strain was transformed with the pTV*PGK-ftt1ΔC(TRP1)* plasmid. Transformants selected on SCD-Trp were sporulated on 2% agar plates containing 1% potassium acetate, 0.1% yeast extract, and 0.05% glucose. At least 20 of the resultant tetrads for each transformation were dissected. All the spores able to grow were analysed by growing patches on YPD plates for three days at 24 °C and replicating onto SCD-Leu and SCD-Ura plates [23] to test if the spore lacks *BMH1* or *BMH2*, respectively.

2.4. Cloning of *ftt1ΔC* and suppression analysis

To isolate *T. reesei* genes involved in protein secretion, a *sec15-1* temperature-sensitive yeast strain HS33-1 [31] was transformed according to Ref. [32] with a *T. reesei* cDNA library [27] in the yeast expression vector pAJ401 with the *PGK1* promoter [28]. The transformants plated on SCD-Ura were grown at the restrictive temperature, 37 °C. Plasmids were isolated from the transformants able to grow in this temperature and transferred to *E. coli* by electroporation for a total yeast DNA preparation.

The plasmid carrying the truncated *ftt1* cDNA, pMS49, was transformed back into *sec15*-strain as well as into various other temperature-sensitive secretion mutant yeast strains (Table 1). Transformants were grown as patches at 24 °C for 3 days, replicated on SCD-Ura-plates and incubated at the restrictive temperature of each strain. The growth was monitored for 3 days. The yeast strains transformed with the empty expression vector pAJ401 were used as negative controls. The growth of transformants obtained with introduction of pAJ401 plasmid carrying the full-length *ftt1* and *ftt2* cDNAs was assayed in a similar manner. Suppression of *sec2-41* and *sec15-1* was tested also by dotting cells as end point dilution series starting with 5×10^7 cells per dot on SCD-Ura-plates, which were incubated at permissive and restrictive temperatures.

2.5. Invertase secretion assay

The *sec2-41* secretion mutant strain was transformed with *T. reesei ftt1ΔC* yeast expression plasmid (pMS49) or the empty vector pAJ401. Transformants were cultivated in SCD-Ura containing 2% glucose in shake flasks at 24 °C overnight. The cultures were diluted into optical density (600 nm) of 0.2, divided in two aliquots, and incubated at 24 and 35 °C. The growth was followed by measurement of optical density (600 nm). After cultivation for 20 h at 35 °C, the growth of *sec2-41* strain expressing *ftt1ΔC* had clearly surpassed the growth of *sec2-41* strain containing the vector plasmid. At 24 °C, these strains grew equally well. The cells cultivated at the restrictive temperature were washed with prewarmed SCD-Ura containing 0.1% glucose, and diluted in this medium into the optical density of 0.5. The cultures were incubated at 35 °C for further 7 h. Cell samples from each culture were collected in 10 mM NaN_3 at 1, 3, and 7 h after derepression of *SUC2* gene. Cells were harvested by centrifugation and resuspended in 0.2 M NaAc buffer pH 5.0.

The wild-type yeast strain (NY13) was transformed with either pMS49, pTV*PGK-ftt1*, pTV*PGK-ftt2* or the empty expression vector pAJ401. Transformants were cultivated in SCD-Ura containing 2% glucose until each culture reached the optical density of 0.5. Cells were then treated as described above.

The invertase activity secreted to the surface was measured using 8×10^7 cell as described [33] using the Glucose/GOD-Perid® method (Boehringer Mannheim GmbH Diag-

A.

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Tharzianum14-3-3        --MGH---EDAVYLAKLAEQAERYEEMVENMKIVASEDRDLTVEERNLLSVAYKNVIGAR 55
TreeseiFTTI             --MGH---EDAVYLAKLAEQAERYEEMVENMKIVASEDRDLTVEERNLLSVAYKNVIGAR 55
LedodesCIP3             MPETR---EDSVYLAKLAEQAERYEEMVENMKRVASSDQELTVEERNLLSVAYKNVIGAR 57
ScerevisiaeBmh1p        --MST-SREDSVYLAKLAEQAERYEEMVENMKTVASSGQELSVEERNLLSVAYKNVIGAR 57
ScerevisiaeBmh2p        --MSQ-TREDSVYLAKLAEQAERYEEMVENMKAVASSGQELSVEERNLLSVAYKNVIGAR 57
CalbicansBMH            --MPA-SREDSVYLAKLAEQAERYEEMVENMKAVASSGQELSVEERNLLSVAYKNVIGAR 57
SpombeRAD24             --MSTTSREDAVYLAKLAEQAERYEEMVENMKSVASTDQELTVEERNLLSVAYKNVIGAR 58
SpombeRAD25             --MSN-SRENSVYLAKLAEQAERYEEMVENMKKVACSNDKLSVEERNLLSVAYKNIIGAR 57
TreeseiFTTII            --MAT-ERESKTFLARLCEQAERYDEMVTYMKEVAQLGGELSVDERNLLSVAYKNVVGTR 57
                                *. .:**:*.******: **  ** **  . .*:*:***********::*:*
                              -------------    -------------       -----------------
                                 α1               α2                    α3

Tharzianum14-3-3        RASWRIVTSIEQKEESKG-NSSQVTLIKEYRQKIENELAKICDDILEVLDQHLIPSAKSG 114
TreeseiFTTI             RASWRIVTSIEQKEESKG-NSSQVALIKEYRQKIEAELAKICDDILEVLDQHLIPSAKSG 114
ScerevisiaeBmh1p        RASWRIVSSIEQKEESKEKSEHQVELICSYRSKIETELTKISDDILSVLDSHLIPSATTG 117
ScerevisiaeBmh2p        RASWRIVSSIEQKEESKEKSEHQVELIRSYRSKIETELTKISDDILSVLDSHLIPSATTG 117
CalbicansBMH            RASWRIVSSIEQKEEAKG-NESQVALIRDYRAKIEAELSKICEDILSVLSDHLITSAQTG 116
SpombeRAD24             RASWRIVSSIEQKEESKG-NTAQVELIKEYRQKIEQELDTICQDILTVLEKHLIPNAASA 117
SpombeRAD25             RASWRIISSIEQKEESRG-NTRQAALIKEYRKKIEDELSDICHDVLSVLEKHLIPAATTG 116
TreeseiFTTII            RASWRIISSIEQKEESKG-SDKHVATIKEYRSKIELELEKVCEDVLNVLDTSLIPNAATG 116
                        ******::*******::  .  :.  *  ** *** **  :..*:* **.  **. * :.
                        ------------        ----------------------------------
                                                        α4

Tharzianum14-3-3        ESKVFYHKIKGDYHRYLAEFAIGDRRKDSADKSLEAYKAATEVAQTELPPTHPIRLGLAL 174
TreeseiFTTI             ESKVFYHKMKGDYHRYLAEFAIGDRRKDSADKSLEAYKAATEVAQTELPPTHPIRLGLAL 174
LedodesCIP3             ESKVFYHKMMGDYHRYLAEFATGDKRKESADKSLEAYKAASDVAVTELPPTHPIRLGLAL 176
ScerevisiaeBmh1p        ESKVFYYKMKGDYHRYLAEFSSGDAREKATNASLEAYKTASEIATTELPPTHPIRLGLAL 177
ScerevisiaeBmh2p        ECKVFYYKMKGDYHRYLAEFSSGDAREKATNSSLEAYKTASEIATTELPPTHPIRLFLAL 177
CalbicansBMH            ESKVFYYKMKGDYHRYLAEFAIAVFRKEAADLSLEAYKAASDVAVTELPPTHPIRLGLAL 176
SpombeRAD24             ESKVFYYKMKGDYYRYLAEFAVGEKRQHSADQSLEGYKAASEIATAELAPTHPIRLGLAL 177
SpombeRAD25             ESKVFYYKMKGDYYRYLAEFTVGEVCKEAADSSLEAYKAASDIAVAELPPTDPMRLGLAL 176
TreeseiFTTII            ESKVFYHKMKGDYHRYLAEFASGEKRKVAATAAHEAYKNATDVAQTELTPTHPIRLGLAL 176
                        *.****:*: ***:******: .   : ::  : *.** *:::* :**.**.*:** ***
                        ------------------    ------------------------    ---------
                                 α5                    α6                    α7

Tharzianum14-3-3        NFSVFYYEILNAPDQACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSSE 234
TreeseiFTTI             NFSVFYYEILNAPDQACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSSE 234
LedodesCIP3             NFSVFYYEILNSPDRACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDM 236
ScerevisiaeBmh1p        NFSVFYYEIQNSPDKACHLRKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDM 237
ScerevisiaeBmh2p        NFSVFYYEIQNSPDKACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDI 237
CalbicansBMH            NFSVFYYEILNSPDRACHLAKQAFDDAVADLETLSEDSYKDSTLIMQLLRDNLTLWT-DL 235
SpombeRAD24             NFSVFYYEILNSPDRACYLAKQAFDEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDA 237
SpombeRAD25             NFSVFYYEILDSPESACHLAKQVFDEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDA 236
TreeseiFTTII            NFSVFYYEILNSPDRACHLAKQAFDDAIAELDSLSEESYRDSTLIMQLLRDNLTLWTSSD 236
                        ********* ::*: **:* **.**:*:::*::***:**:***************** .
                        ------          -------------              --------------
                                              α8                        α9

Tharzianum14-3-3        AETPARLMPLLRRR---PLLRL---PSRRRRAQG------- 262
TreeseiFTTI             AETSAGQVEAPPKEDTPAEAAA---PAEEPKAE-------- 264
LedodesCIP3             QDSADKPAEK------DEAADA---PADE------------ 256
ScerevisiaeBmh1p        SESGQAEDQQQQQQHQQQQ------PPAAAEGEAPK----- 267
ScerevisiaeBmh2p        SESGQEDQQQQQQQQQQQQQQQQQQAPAEQTQGEPTK----- 273
CalbicansBMH            SEAPAATEEQQQS--SQAP------AAQPTEGKADQE---- 264
SpombeRAD24             EYSAAAAGG-NTEGAQENA------PSNAPEGEREPKATHR 271
SpombeRAD25             EYNQAKEEAPAAAAASENE------HPEPKESTTDTVKA-- 269
TreeseiFTTII            SGEAEQAGEAKKDEGEAAKPAEEEPKAEEPAPEATS----- 272
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Fig. 1. (A) A multiple sequence alignment (Clustal W 1.81) of fungal 14-3-3 proteins. Asterisks indicate conserved residues and dots indicate residues that share similar biochemical properties. Dashed lines under the sequence blocks indicate the positions of putative alpha helices in the three-dimensional structure. Amino acid blocks in boldface indicate areas important for target protein binding. The arrow marks the site of *ftt1* truncation. (B) A pairwise (Blast2) comparison of selected 14-3-3 homologues. The specific names are abbreviated as follows: *Trichoderma reesei* = Treesei, *Trichoderma harzianum* = Tharzianum, *Lentinus edodes* = Ledodes, *Saccharomyces cerevisiae* = Scerevisiae, *Schizosaccharomyces pombe* = Spombe, *Candida albicans* = Calbicans.

B.

IDENTITY	Treesei FTTII	Tharzianum 14-3-3	Ledodes CIP3	Scerevisiae Bmh1p	Scerevisiae Bmh2p	Spombe RAD24	Spombe RAD25	Calbicans BMH
Treesei FTTI	75%	**98%**	85%	79%	81%	82%	76%	82%
	Treesei FTTII	75%	76%	74%	73%	73%	71%	74%
		Tharzianum 14-3-3	87%	80%	80%	82%	75%	80%
			Ledodes CIP3	82%	82%	83%	77%	85%
				Scerevisiae Bmh1p	**98%**	73%	77%	83%
					Scerevisiae Bmh2p	78%	75%	82%
						Spombe RAD24	74%	72%
							Spombe RAD25	77%

Fig. 1 (*continued*).

nostica, Germany). Invertase activity was calculated by using the definition where one unit of invertase liberates 1 μmol of glucose in 1 min at 30 °C.

2.6. α-Amylase secretion assay

The *sec2-41* strain was transformed with the plasmid Yepαa6 [34], in which *Bacillus amyloliquefaciens* α-amylase is expressed from a modified *ADH1* promoter. The strains were then transformed with the plasmids pMS49 or pAJ401. Transformants were cultivated in buffered SCD-Ura containing 2% succinic acid and 10 mM $CaCl_2$, pH 6 [35] in shake flasks at 24 °C overnight. The cultures were diluted into optical density (600 nm) of 0.2, divided in two 50 ml aliquots and incubated at 24 and 35 °C. Five medium samples were collected from which the α-amylase activity was assayed as described [34].

2.7. Western blotting

The total amount of approximately 20 μg of proteins in the cell lysate were separated in SDS-PAGE and transferred to Hybond P membrane (Amersham). The 14-3-3 proteins were recognised with polyclonal rabbit antibody raised against yeast Bmh1p (a gift from H.Y. Steensma, Leiden University, The Netherlands). The primary antibody was detected by horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad). The activity of horseradish peroxidase was detected with the ECL Western blotting analysis system (Amersham).

2.8. Log phase growth assay

To examine whether the expression of truncated *ftt1* has a dominant effect on growth of wild-type yeast, NY13 strain was first transformed with pTV*PGK-ftt1,* pTV*PGK-ftt2*, pMS49 expression plasmids or the empty plasmid pAJ401. The transformants were cultivated at 30 °C overnight. The overnight cultures were diluted into optical density (600 nm) of 0.2 and incubated in SCD-Ura (2% glucose) at 30 °C. Samples for optical density measurement and cell number counting were taken approximately every third hour. The cultures were maintained in log phase by diluting them 1:10 whenever the culture with most vigorous growth approached the optical density of 2.0.

3. Results

3.1. Isolation of two 14-3-3 genes from T. reesei

A *T. reesei* cDNA library in a multicopy yeast expression plasmid was introduced into the temperature-sensitive *S. cerevisiae* mutant strain *sec15-1* in order to isolate *T. reesei* genes whose overexpression is capable of either complementation or suppression of this late-acting secretory mutation. Several clones were able to suppress the growth defect of *sec15-1* at the restrictive temperature, 37 °C. The cDNA insert of one of these clones (pMS49) encoded a 14-3-3-like protein. Sequence comparison of this cDNA with known 14-3-3 proteins revealed that it was not full length but truncated at an internal *Xho*I restriction site, and it lacked 115 amino acids from its C-terminus. Truncation of this cDNA at the *Xho*I site may have been caused by the cDNA library construction strategy that includes a *Xho*I digestion at a linker in the 3′ ends of the cDNAs. The internal *Xho*I sites are normally protected by methylation [27].

The corresponding full-length cDNA was isolated from the cDNA library by using the truncated cDNA as a hybridisation probe. Ten positive plaques were isolated and sequencing revealed that three of them were full-length clones coding for the same 14-3-3-like protein as the

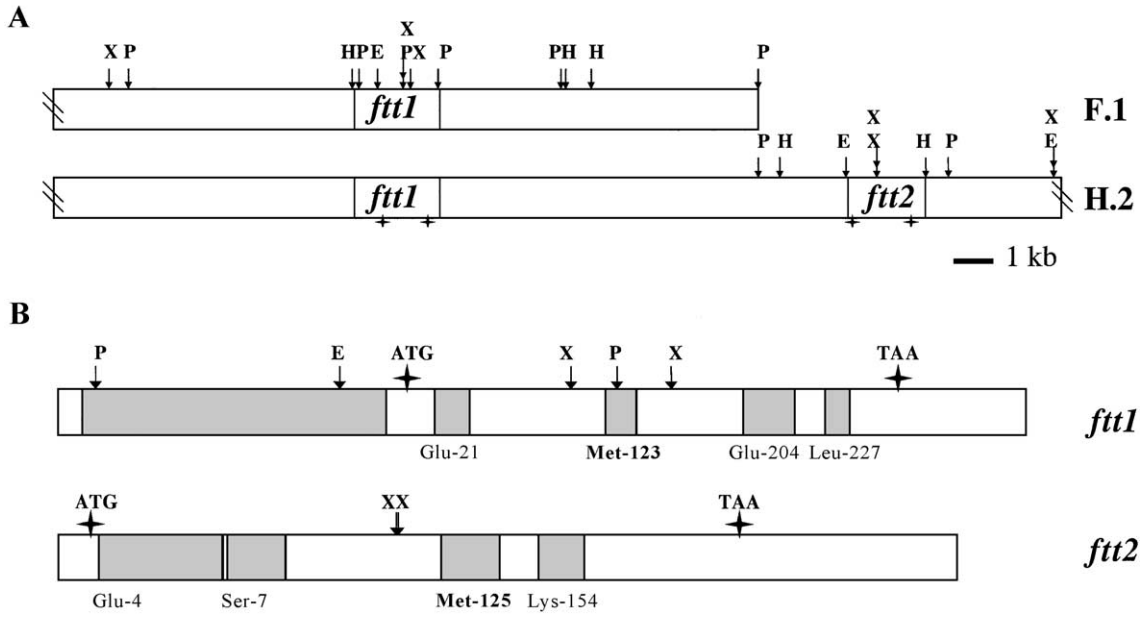

Fig. 2. (A) A diagram of *T. reesei ftt1* and *ftt2* gene localisation in F.1 and H.2 cosmids based on Southern hybridisation analysis. The recognition sites of restriction enzymes *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), and *Xho*I (X) are marked with arrows. The coding region of each gene is located between asterisks. (B) A diagram of *T. reesei ftt1* and *ftt2* gene structures. The introns are shaded and the site of each intron is indicated by the corresponding amino acid. The amino acids in boldface denote the conserved intron location. The coding region of each gene lies between asterisks. The recognition sites of restriction enzymes *Eco*RI (E), *Pst*I (P), and *Xho*I (X) were indicated by arrows.

truncated cDNA isolated before. Two other full-length cDNAs were identical to each other but different from the previously isolated cDNA, although they clearly coded for a 14-3-3-like protein as well. We named these cDNAs as follows: *ftt1* (*f*ourteen *t*hree *t*hree) for the full-length cDNA first isolated, *ftt1*Δ*C* for the truncated version, and *ftt2* for the second cDNA. Pairwise comparison of amino acid sequences revealed that FTTI was very close to its counterpart isolated from *Trichoderma harzianum* (98% identity) (Fig. 1B) while FTTII was quite distinct when compared with other fungal 14-3-3 proteins known. The amino acid sequence of FTTII differs from the others in putative alpha helices 1 and 2 and 6 mainly by conservative amino acid replacements (Fig. 1A). It is remarkable that also the 3′ flanking nucleic acid sequences of *T. reesei ftt1* and *T. harzianum 14-3-3* share as high as 86% identity. No other homologies within the 3′ flanking region were found for either of the *T. reesei ftt* genes.

The genomic *ftt1* and *ftt2* genes were isolated from a *T. reesei* cosmid library. The positive colonies were found in hybridisation with ^{32}P-labelled *ftt1*Δ*C*. These colonies contained two different types of cosmids. Restriction analysis and Southern hybridisation of the two cosmids (F.1 and H.2) revealed that they were partially overlapping and that one of them (H.2) contained both *ftt* genes, and the other (F.1) carried only the *ftt1* gene. The distance between the two *ftt* genes, as estimated on the basis of Southern digestion and hybridisation patterns, appeared to be approximately 11 kb (Fig. 2A). The sequence analysis of the *ftt* genes revealed that the *ftt1* gene (EMBL accession number AJ297910) has four introns in the coding region and one exceptionally long (682 bp) intron at the 5′ flanking region. The *ftt2* gene (EMBL accession number AJ297911) has also four introns

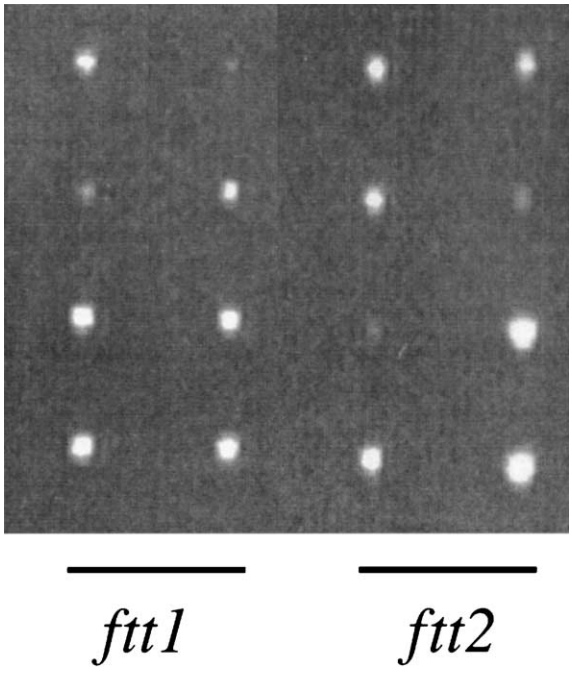

Fig. 3. Complementation of yeast *bmh1 bmh2* double disruption with *T. reesei ftt1* and *ftt2* cDNAs. A heterozygous diploid strain *bmh1*::*LEU2*/+*bmh2*::*URA3*/+was transformed with plasmid expressing either *ftt1* or *ftt2*. The transformants were sporulated and tetrads were dissected. Each column represents one dissection.

in the coding region but none at the 5′ flanking region. Only one of the introns in both *ftt* genes was at a conserved location (Fig. 2B).

To verify that there are only two *ftt* genes in *T. reesei*, Southern hybridisation analysis of genomic DNA was performed under stringent and nonstringent conditions with *ftt1ΔC* cDNA as a probe. Similar results were obtained in all conditions: two hybridising bands could be detected when the genomic DNA was digested with *Eco*RI or *Hin*dIII, three bands with *Pst*I and four with *Xho*I (data not shown). This detection is in accordance with the number of cleavage sites of each restriction enzyme in the two *ftt* genes. There were two recognition sites for *Xho*I in *ftt1* and *ftt2*. In *ftt2* gene, the two *Xho*I sites are very close to each other, and therefore the intervening fragment originating from *Xho*I digestion could not be detected. Thus, four distinct bands instead of five emerged (see Fig. 2). The Southern hybridisation pattern suggests that *T. reesei* genome does not have other genes closely related to *ftt1* and *ftt2*.

3.2. Both T. reesei ftt genes can complement the yeast bmh1 bmh2 double disruption

To test whether the isolated *T. reesei ftt1* and *ftt2* genes are functional counterparts of the yeast *BMH1* and *BMH2* genes, a complementation assay was carried out. For that experiment, either *ftt1*, *ftt2*, or *ftt1ΔC* coding region in an expression vector or the empty vector as negative control was transformed into the yeast strain GG583. This diploid yeast strain has one active and one disrupted copy of the genes *BMH1* and *BMH2*. *BMH1* and *BMH2* have been disrupted in this strain by fragments containing either the *LEU2* gene or the *URA3* gene, respectively. Double disruption of both the *BMH1* and *BMH2* genes is lethal and single disruptions are viable [36]. Thus, if spores lacking both of the *BMH1* and *BMH2* genes but expressing *ftt1*, *ftt2*, or *ftt1ΔC* are viable, complementation of lethality has occurred. The transformants obtained in transformation with *T. reesei ftt1* or *ftt2* under the control of the yeast *ADH1* promoter or with *ftt1ΔC* under the control of the yeast *PGK1* promoter in multicopy plasmids were subjected to tetrad analysis. The transformation with the *ftt1ΔC* expression plasmid gave two or three viable spores, similarly as the transformation with the vector alone. The majority of tetrads from transformations with the *ftt1* or *ftt2* plasmids gave four viable spores. However, one of the spores from each tetrad from the latter transformations grew markedly slower than the other three spores (Fig. 3). The analysis of the spores revealed that the spore giving rise to a slowly growing colony was able to grow on plates lacking both leucine and uracil and thus lacked both *BMH1* and *BMH2* genes. The expression of either *ftt1* or *ftt2* cDNA thus appeared to complement the yeast double disruption although not to the full extent, as suggested by the slower growth. There was no apparent difference in the complementation ability between *T. reesei ftt1* and *ftt2*. The expression of *ftt1ΔC*, instead, could not complement the yeast *bmh1 bmh2* disruption.

3.3. The C-terminally truncated ftt1 can suppress the growth of several late-acting secretory mutations of S. cerevisiae

The C-terminally truncated *ftt1* of *T. reesei* was isolated as a multicopy suppressor of yeast temperature sensitive secre-

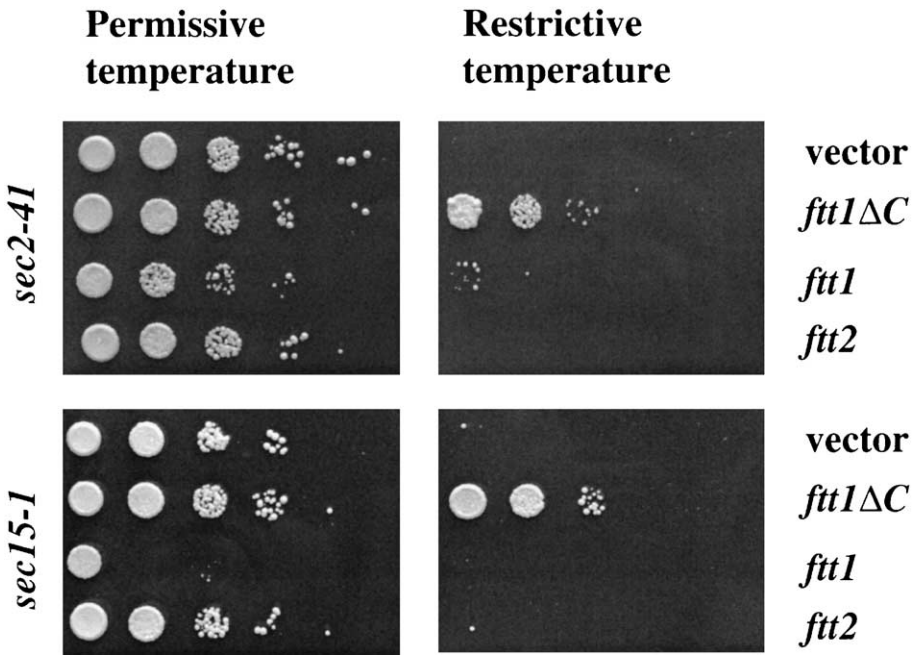

Fig. 4. Effects of *T. reesei ftt1ΔC* expression on growth of yeast mutant strains *sec2-41* and *sec15-1* at permissive and restrictive temperatures. Transformants were applied on the SCD-Ura-plates as dilution series.

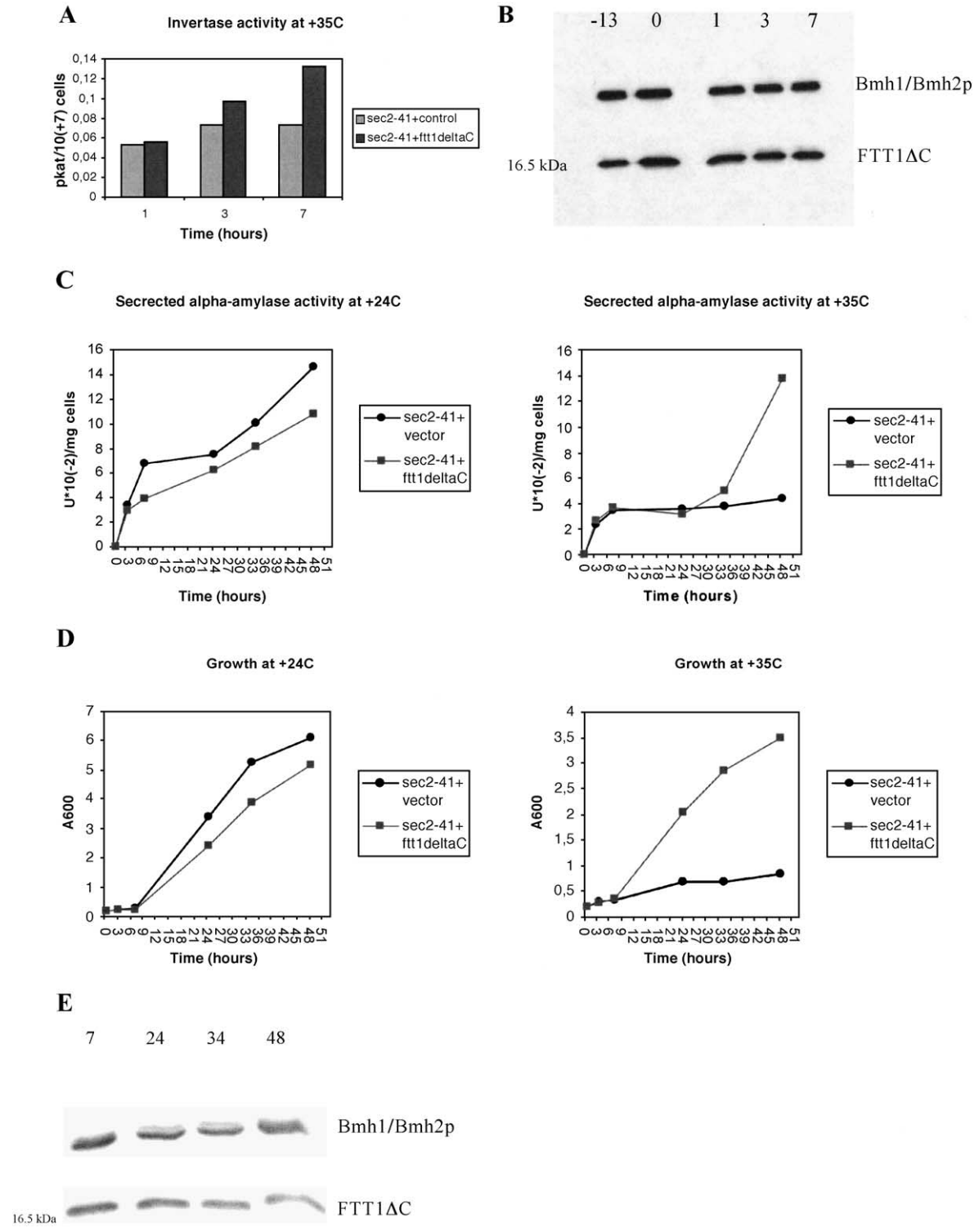

Fig. 5. (A) Effect of *T. reesei ftt1ΔC* expression on invertase secretion of the yeast *sec2-41* mutant strain at the restrictive temperature. The time after transfer to derepressing medium is shown. (B) A Western blot analysis of the *T. reesei* FTTIΔC protein level in *sec2-41* cells used for the invertase assay (Panel A). Equal amounts of total yeast proteins in the cell lysates were loaded for each time point. (C) Effects of *T. reesei ftt1ΔC* expression on *Bacillus* α-amylase secretion of *sec2-41* strain at the permissive and restrictive temperatures. (D) Effects of *T. reesei ftt1ΔC* expression on growth of *sec2-41* at the permissive and restrictive temperatures. (E) A Western blot analysis of the *T. reesei* FTTIΔC protein level in *sec2-41* cells at the restrictive temperature of +35 °C grown for the α-amylase determination (Panels C and D).

tory mutation *sec15-1* as described above. Consequently, we tested if overexpression of this truncated version, *ftt1ΔC*, could rescue the growth of a number of other late-acting secretory mutations. The multicopy expression plasmid with *ftt1ΔC* (pMS49) or the empty vector (pAJ401) were transformed into the yeast mutant strains and the growth of the transformants was assayed at the restrictive temperature of each temperature-sensitive mutant strain. In addition to *sec15-1* mutation, the mutations *sec2-41*, *sec3-101, sec7-1* were also clearly suppressed (Fig. 4) whereas the growth of the strains *sec1-1*, *sec1-11* and *sec4-8*, *sec9-4*, and *sec19-1* was weakly improved (data not shown). The growth of the strains having *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, *sec17-1*, *sec18-1* or *sso2-1* mutations was not rescued. The suppression of the same yeast secretory mutants was tested with the full-length *ftt1* and *ftt2* cDNAs in a similar manner as done with *ftt1ΔC*. The overexpression of full-length *ftt* cDNAs did not suppress the growth defects of any of these yeast strains. In contrast, overexpression of *ftt1* slightly retarded the growth of *sec15-1* at the permissive temperature.

3.4. The C-terminally truncated ftt1 suppressed the secretion defect of sec2-41

To test whether the *ftt1ΔC* suppresses not only the temperature-sensitive growth defect but also the secretory defect, secretion of invertase and a heterologous reporter protein *B. amyloliquefaciens* α-amylase was monitored at the permissive and restrictive temperatures in *sec2-41* strain carrying the *ftt1ΔC* expression plasmid (pMS49) or the empty vector (pAJ401). *Bacillus* α-amylase was expressed from a second expression plasmid from the modified yeast *ADH1* promoter. Overexpression of *ftt1ΔC* in *sec2-41* improved growth at the restrictive temperature 35 °C (see Materials and methods) and enhanced secretion of invertase (Fig. 5A), expression of which was released from glucose repression by shifting the cells into low glucose medium. A low and equal level of invertase was detected in both cells 1 h after derepression. At the next time points, the invertase activity was increased and there was also a difference in the activity level between the *sec2-41* cells overexpressing *ftt1ΔC* or vector control (Fig. 5A). The difference in invertase activity was approximately 1.8-fold at 7 h after derepression. Similarly, the secretion of heterologous α-amylase was enhanced by *ftt1ΔC* overexpression in *sec2-41* strain at the restrictive temperature (Fig. 5C). However, the effect was clearly delayed. The growth suppression of *sec2-41* by overexpression of *ftt1ΔC* was detectable after cultivation of 24 h (Fig. 5D) but suppression of secretion required longer incubation (Fig. 5C). To exclude the possibility that this would be caused by changes in the FTT1ΔC protein level during the culture, Western blotting with an antiserum against yeast Bmh1p was done. This antiserum could readily detect the expressed FTT1ΔC in approximately equal amounts at all time points from lysates of *sec2-41* cells expressing *ftt1ΔC* (Fig. 5B,E). The antiserum recognised only endogenous Bmh1p/Bmh2p from lysates of *sec2-41* cells harboring empty expressing vector (data not shown). At the permissive temperature, overexpression of *ftt1ΔC* slightly decreased the secretion of α-amylase (Fig 5C). However, this effect was not found when another secretory mutant strain, *sec15-1*, was tested in a similar manner (data not shown). It appeared that *sec2-41* and *sec15-1* mutant strains expressing the *T. reesei ftt1ΔC* cDNA were able to secrete both the heterologous α-amylase and the endogenous invertase at permissive and restrictive temperatures.

3.5. Overexpression of ftt1 enhanced invertase secretion in wild-type yeast

Since overexpression of *T. reesei ftt1ΔC* could suppress the secretion defect of two yeast secretory mutants (Figs. 4 and 5A), we wanted to test if overexpression of full-length *ftt1* or *ftt2* would have any effect on secretion in wild-type yeast. The invertase secretion assay, carried out in similar way as with *sec2-41* strain (Fig. 5A), showed that overexpression of *ftt1* in a wild-type strain clearly enhanced secretion (Fig. 6). The difference in invertase activity levels between vector control and *ftt1* was most noticeable, 2.8-fold, at 3 h after transfer to low glucose medium. The full-length *ftt2* or *ftt1ΔC* did not have a clear effect on invertase secretion (Fig. 6).

3.6. Both the C-terminally truncated ftt1 and full-length ftt1 retarded growth of wild-type yeast

Roth et al. [21] generated fragments covering various domains of yeast Bmh1p and Bmh2p to overexpress them

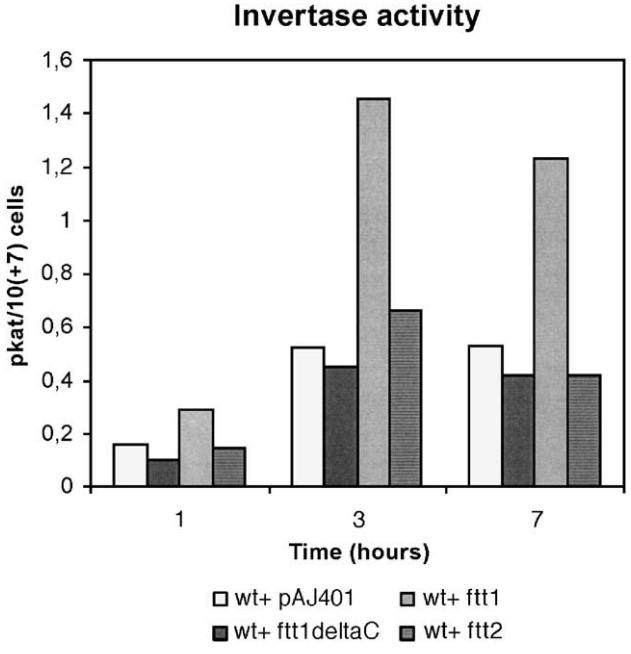

Fig. 6. Effect of *T. reesei ftt1* expression on invertase secretion of a wild-type yeast strain.

in yeast. The fragments of Bmh2p that are supposed to be involved in dimer formation or effector protein binding [37] appeared to have dominant-negative effect on growth. These fragments were suggested to interfere with the function of endogenous Bmh proteins [21]. The overexpression of *ftt1*Δ*C* appeared to slow down the growth of *sec2-41* strain at the permissive temperature (Fig. 5D). To examine whether the expression of *ftt1*Δ*C*, *ftt1* or *ftt2* have dominant effects on growth, a wild-type yeast strain, NY13, was transformed with plasmids expressing these *T. reesei* genes or with the vector pAJ401. The transformants were cultivated so that the cells were kept in the logarithmic growth phase for a long period. The experiment showed that overexpression of *ftt1*Δ*C* retarded the growth of NY13 during log phase growth (Fig. 7). However, this is probably not a dominant effect due to truncation of *ftt1*Δ*C* since overexpression of full-length *ftt1* also retarded growth (Fig. 7). Additionally, overexpression of *ftt1* resulted in expanded cell morphology (data not shown). Overexpression of *ftt2* enhanced the growth of wild-type yeast (Fig. 7).

3.7. ftt expression is not regulated according to growth rate in T. reesei

The mammalian and yeast 14-3-3 proteins have been implicated in cell cycle control and signal transduction pathways affecting growth [38]. Therefore, it was of interest to examine whether the *T. reesei ftt1* and *ftt2* genes are regulated according to the growth rate in *T. reesei*. This was studied by Northern hybridisation from lactose-limited chemostat cultures with six different dilution rates ranging from 0.02 to 0.07 l/h. To avoid cross-hybridisation between *ftt1* and *ftt2*, the probe fragments were made by PCR from the noncoding 3′ flanking regions of the two genes. The *ftt1* probe hybridised with a band of 1.35 kb and the *ftt2* probe with three bands of 1.9, 1.65 and 1.43 kb in length (Fig. 8). Both *ftt1* and *ftt2* mRNAs showed very little variation between samples from different cultures and thus these genes appear not to be regulated at the transcriptional level according to the growth rate. This is consistent with results obtained for *T. harzianum Th1433*, which was constitutively expressed except that soon after spore germination, the expression level was higher compared to that of later time points [3].

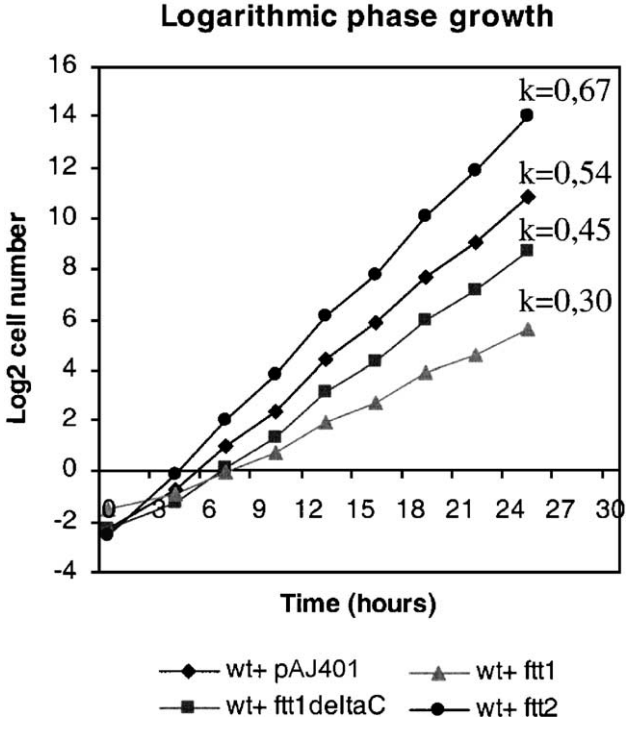

Fig. 7. A log2 plot of cell number of a wild-type yeast strain transformed with the expression vectors of *ftt1*, *ftt2*, *ftt1*Δ*C* or the empty vector pAJ401. Cultures were maintained in logarithmic phase growth by dilution. The slope for each curve is indicated.

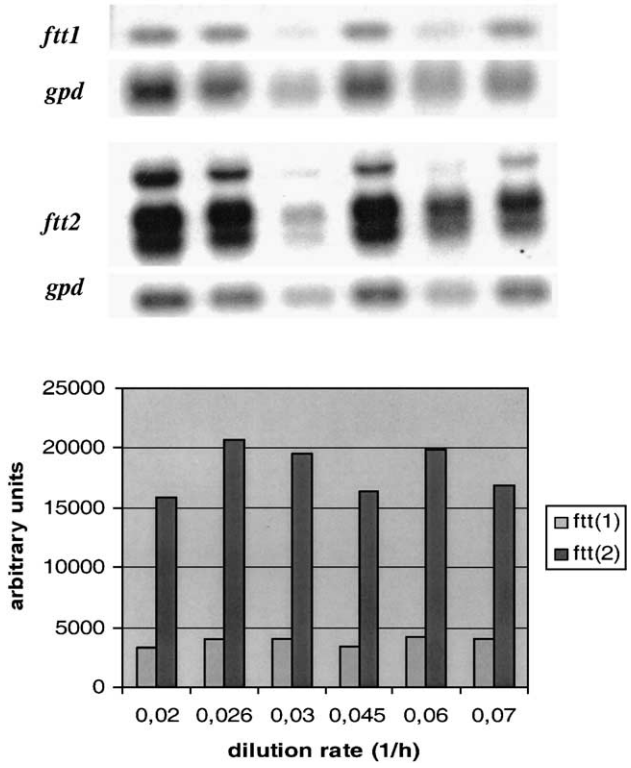

Fig. 8. Northern analysis of lactose-limited chemostat cultures of *T. reesei* with different dilution rates. Hybridisation was performed with PCR-derived fragments from 3′ flanking sequences of *ftt1* and *ftt2*. The *gpd*-normalised intensities of *ftt1* and *ftt2* signals are shown below.

4. Discussion

Although multiple isoforms of 14-3-3 proteins exist in mammals and plants, two isoforms would appear to prevail in fungi: Bmh1p and Bmh2p have been characterised for *S. cerevisiae* [36] and Rad24 and Rad25 for *Schizosacchar-*

omyces pombe [4]. Furthermore, according to Southern analysis, there appears to be two genes encoding 14-3-3-like proteins also in the filamentous fungus *T. harzianum* [3]. In this work, we have isolated two *14-3-3* genes from *T. reesei*, and based on Southern data, we suggest that there would be only these two genes encoding 14-3-3-like proteins in the *T. reesei* genome. A basidiomycete, *Lentinus edodes*, could be an exception for the rule as, according to a Southern hybridisation in a recent report, it would have only one *14-3-3* gene [39].

The *T. reesei ftt1* gene is highly similar with the isolated *T. harzianum 14-3-3* gene, with 98% identity at amino acid level, and 86% identity in the 3′ flanking region. A similar high conservation of the 3′ untranslated regions was found between the ε isoforms of human, rat and mouse *14-3-3* genes [40], and ζ isoforms of *Rana rugosa* and human [41]. It has been proposed that the 3′ flanking sequence of human 14-3-3ε overlaps with the 5′ region of LIS-1 gene [40]. The 3′ noncoding region of *ftt1* did not, however, show significant similarity with any other sequence than *T. harzianum 14-3-3*. The *T. reesei ftt2* gene was relatively distinct from its fungal equivalents; its closest homologues were *ftt1* of *T. reesei* and the *14-3-3* gene of *T. harzianum*. In Southern analysis of *T. harzianum* genomic DNA [3], the putative second gene encoding 14-3-3 protein gave a relatively weak signal implying deviation of this gene from the known *14-3-3* gene. It is possible that an uncovered *14-3-3* gene of *T. harzianum* resembles more *ftt2* than *ftt1* of *T. reesei*.

Both of the isolated *T. reesei ftt* cDNAs were able to complement the yeast *bmh1 bmh2* double disruption (Fig. 3). The complementation was not complete since the disruptant with either *ftt1* or *ftt2* expression grew clearly more slowly than the strains with one of the yeast *BMH* genes intact. It was somewhat unexpected that *T. reesei ftt1* and *ftt2* could complement the yeast disruption with approximately equal efficiency as *ftt1* is clearly more similar to the yeast *BMH1* and *BMH2* than *ftt2* (Fig. 1B). The complementation experiment indicates that, despite its sequence divergence from other fungal 14-3-3 proteins, FTTII can substitute for the cellular functions whose lack causes lethality in the *bmh1 bmh2* disruption. In contrast, the C-terminally truncated *ftt1* could not complement the yeast *bmh1 bmh2* disruption. This was expected since *ftt1ΔC* lacks part of its putative effector binding region. Complementation of the same yeast disruptions has been tested with six *Arabidopsis 14-3-3* genes, and four of them could complement the disruptions [42].

The growth and secretion of fungal species is highly polarised. In yeast, this polarisation is based on the delivery of secretory vesicles along actin filaments into the growing bud. The multiprotein complex called exocyst forms a bridge connecting the secretory vesicle to the site of exocytosis on plasma membrane [43]. Our aim was to clone by complementation the counterpart of a central component of the yeast exocyst complex, Sec15p, from the filamentous fungus *T. reesei*. In this assay, we obtained as a suppressor a cDNA (*ftt1ΔC*) coding for a C-terminally truncated 14-3-3-like protein. It was also noticed in this work that *ftt1ΔC* suppressed in addition to *sec15-1*, also the growth defect of *sec2-41*, *sec3-101*, and *sec7-1* mutants. Out of these mutated proteins suppressed by FTT1ΔC, Sec15p and Sec2p are located on surface of the secretory vesicle in yeast [43,44]. Sec2p acts as the guanine nucleotide exchange factor (GEF) and Sec15p is an effector of the Sec4p [45,43], also located on the secretory vesicle [43]. Sec4p controls the assembly of the exocyst, which links the vesicle to the plasma membrane [43] at a site marked by Sec3p [46]. All the yeast genes suppressed by *ftt1ΔC* except *SEC7* share genetic interactions with *SEC4* [47–49]. However, Sec7p has been proposed to be required for vesicle fusion as well, although at the ER–Golgi stage [50].

Since yeast growth and secretion are intimately connected, we tested if secretion in these secretion-deficient yeast strains was also rescued by *ftt1ΔC* overexpression. It appeared that invertase and α-amylase secretion defect of *sec2-41* was suppressed in liquid cultures, although somewhat delayed (Fig. 5). In addition, although the full-length *ftt1* was unable to suppress the growth defect of any of the secretory mutant strains tested, overexpression of it could enhance the invertase secretion of a wild-type yeast strain (Fig. 6).

The 14-3-3 proteins are extremely well conserved throughout evolution, are involved in numerous cellular processes, and have multiple target proteins. Based on the crystal structure of a mammalian 14-3-3 protein [37], each monomer of these dimeric proteins is composed of nine antiparallel α-helices. Four parallel helices (α3, α5, α7, and α9) form an amphipathic inner groove. A cluster of charged and polar residues of helices 3 and 5 outline one side of the groove, and a cluster of hydrophobic residues of helices 7 and 9 forms the other side of the groove. The functional organisation of the 14-3-3 proteins is not thoroughly known but the interaction between 14-3-3 proteins and their target proteins is suggested to occur between discrete phosphoserine-containing motifs of the target [51,52] and the amphipathic groove of 14-3-3 proteins [37]. The interacting residues in this groove are conserved in all 14-3-3 isotypes [53] and can also be identified in the *T. reesei* FTTI and FTTII proteins (Fig. 1A).

Suppression of several yeast *sec*-mutations functioning at exocytosis by the truncated FTTI and enhancement of secretion of wild-type yeast by FTTI suggest that 14-3-3 proteins may be involved in the control of exocytosis in fungi. It has been shown before that overexpression of the yeast *BMH2* suppresses mutations in the clathrin heavy-chain gene, *CHC1* [20] and overexpression of Bmh2p C-terminal fragment causes defects in actin organisation and polarised targeting of the vesicles [21]. However, more direct function for 14-3-3 proteins in docking/fusion of secretory vesicles with plasma membrane has not been

reported. The *Trichoderma ftt*Δ*C* lacks almost half of the coding region and thus the truncated FTTI protein lacks the entire hydrophobic sequence, which in the mammalian 14-3-3 protein [37] forms one side of the amphipathic groove. Most of the mutational and crystallisation studies have shown that the basic cluster of the groove is important for the interaction of 14-3-3 proteins with diverse ligands (Ref. [13] and references therein). If the fungal 14-3-3 proteins play a role in exocytosis, one way to explain the pattern of the suppressor activities could be that the wild-type protein is unable to interact with a mutated Sec protein(s) that may be structurally distorted. In the truncated form, the binding is restored due to its altered size or binding properties caused by the deletion. On the other hand, the wild-type FTTI may interact with the wild-type protein(s) and thus enhance secretion. The binding motifs in the yeast 14-3-3 target proteins are not known but at least the Sec proteins in question do not contain a clear phosphoserine motif typical for mammalian 14-3-3 target proteins [51,52]. It is also possible that the suppression of the *sec* mutations occurs by another mechanism than direct binding between the Sec and 14-3-3 proteins. The overexpression of both the truncated and full-length *ftt1* retarded the growth of wild-type yeast at log phase (Fig. 7). Hence, they may interfere with the function of endogenous BMH proteins in cell polarity through, e.g. the stress response pathway [54,55].

Interactions of 14-3-3 proteins with the secretory pathway in yeast and mammalian cells have been observed in several studies [14–21]. In addition, certain isoforms of mammalian 14-3-3 proteins have been reported to bind phospholipids [56] or synaptic membranes [57], and 14-3-3 proteins have been also found to colocalise with trans-Golgi network-derived vesicles in mammalian epithelial cells [58], and with *Drosophila* synaptic vesicles [17]. It was also suggested that 14-3-3 proteins could facilitate the association of kinesin-like motor protein with the membranes of the Golgi complex or transported vesicles [18]. Furthermore, the *SNC1* gene encoding a yeast exocytotic vesicle-SNARE, was isolated by its ability to suppress the loss of C-terminal function of adenylate cyclase-associated protein (CAP) [59], and a 14-3-3 protein has been reported to interact with a similarly truncated CAP in *L. edodes* and *S. pombe* [39]. As many of these studies suggest association of 14-3-3 proteins with post-Golgi vesicles, it is tempting to speculate that *T. reesei ftt1*Δ*C* would operate in connection with the secretory vesicles when suppressing the yeast secretory mutations. This interpretation is supported by the fact that besides Sec4p, Sec2p and Sec15p are also associated with the post-Golgi vesicles [43,45].

The precise mode of action of 14-3-3 proteins in vesicle trafficking is not presently known. However, our results presented in this paper should provide an additional piece of evidence for a functional role of the 14-3-3 proteins in the secretory process.

Acknowledgements

The authors wish to thank Riitta Nurmi for skilful technical assistance. We thank P. Brennwald, P. Haarer, P. Novick, and R. Schekman for providing us with *sec* mutants, and Benjamin Hall for pYcDE-2 plasmid. P. van Heusden and H. Y. Steensma are acknowledged for *bmh1 bmh2* double disruption yeast strain and antibody against Bmh1p. This work is a part of the research programme "VTT Industrial Biotechnology" (Academy of Finland; Finnish Centre of Excellence programme, 2000–2005, Project No. 64330). It was also supported by a grant from the Viikki Graduate School of Biosciences.

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